

REMARKS

The Office Action has been reviewed carefully and claims 1, 2, 3, 11 and 29 have been amended in a sincere effort to place the application in condition for allowance. Claim 28 has been canceled. New claims 30-32 have been added. Support for the language “segment” can be found at page 4, line 2 *et seq.* of the specification. Support for the language “vascular tissue” can be found at page 4, line 10 *et seq.* of the specification. No new matter has been added. In view of the foregoing amendments and following remarks, Applicant believes that all the rejections are in condition for withdrawal and that claims 1-3, 5, 7-13, 19, 23-25, 26, 27 and 29-33 are in condition for allowance.

The Claimed Invention

The claimed invention as claimed in independent claim 1 is directed to a method for delivering a chemical or biological entity to the surface of a segment of vascular tissue of a patient. The method comprises binding a molecule to the surface of the vascular tissue segment, wherein the molecule includes at least one reactive group that reacts with groups present on the surface of the vascular tissue segment and at least one signaling molecule; attaching the chemical or biological entity to the signaling molecule by means of a recognition molecule, wherein the recognition molecule is specific for the signaling molecule, wherein the recognition molecule and the signaling molecule have an affinity for each other; wherein the reactive group binds covalently to the surface of the vascular tissue segment; and wherein the delivery steps can be effected under conditions tolerable *in vivo*.

Additionally, the claimed invention as claimed in new independent claim 30 is directed to a method for delivery of a chemical or biological entity to a segment of vascular tissue of a patient, comprising covalently binding a molecule comprising a reactive molecule and a signaling molecule, in which the molecule comprises N-hydroxy-succinimide-biotin, the reactive molecule comprises N-hydroxy-succinimide, and the signaling molecule comprising biotin, and wherein the N-hydroxy-succinimide reacts with groups present on the vascular tissue segment; attaching the chemical or biological entity to the biotin signaling molecule by means of a recognition molecule comprising avidin, wherein the avidin recognition molecule and the biotin signaling molecule have an affinity for each other; and wherein the delivery steps can be effected under conditions tolerable *in vivo*.

Claim Objections

Claim 11 is objected to because the recitation “solid particulate” assertedly is redundant. Claim 11 has been amended to delete the term “solid.” Applicants respectfully request withdrawal of this objection.

Claim 28 is objected to under 37 C.F.R. 1.75(c) as being of improper dependent form for failing to further limit the subject matter of a previous claim. Claim 28 has been canceled, and thus this objection is moot.

35 U.S.C. § 112, Second Paragraph, Rejection

Claim 29 is rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. The Examiner asserts that it is not clear whether the term “compatibility” in the claim refers to compatibility with the host or compatibility with the recognition molecule. Claim 29 has been amended to clarify that “compatibility” refers to compatibility between the signaling molecule and the recognition molecule.

35 U.S.C. § 112, First Paragraph, Rejection

Claims 1-3, 5, 7-12, 19, 24 and 26-28 are rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. The Examiner asserts that the specification is enabling only for a method for a two-step delivery of a chemical or biological entity to cellular components of a blood vessel of a patient, wherein the cellular components are blood cells and endothelial cells and wherein the method comprises binding of NHS-biotin to blood cells or endothelial cells, followed by the attachment of a chemical/biological entity/avidin conjugate.

Claims 1, 2 and 3 have been amended to recite that a chemical or biological entity is delivered to the surface of a segment of vascular tissue which has been modified by the covalent binding of a molecule comprised of a reactive group and a signaling molecule. Applicants submit that claims 1, 2 and 3 as amended are more than adequately enabled by the specification. Additionally, new claims 30-32 have been added which recite a method for delivering a chemical or biological entity to a segment of vascular tissue of a patient, comprising covalently binding a molecule comprising a reactive molecule and a signaling molecule, in which the molecule comprises N-hydroxy-succinimide-biotin, the reactive molecule comprises N-hydroxy-succinimide and the signaling molecule comprises biotin, wherein said N-hydroxy-succinimide reacts with groups present on the vascular tissue segment; attaching the chemical or biological entity to the biotin signaling molecule by means of a recognition molecule comprising avidin, wherein the avidin recognition molecule

and the biotin signaling molecule have an affinity for each other; and wherein the delivery steps can be effected under conditions tolerable *in vivo*.

Applicants respectfully point out that, contrary to the Examiner's assertion that the cellular components that are targeted by chemical or biological entities are circulating blood cells, the cellular components of the claimed invention are stationary segments of vascular tissue, such as endothelial cells. To further substantiate the nature of the cellular components of the claimed invention and to provide evidence that the present invention as now claimed is more than adequately enabled, Applicants submit concurrently herewith an article authored by the Applicants (Deglau, T.E. et al., "Targeting Microspheres and Cells to Polyethylene Glycol-Modified Biological Surfaces," published online: December 19, 2006, Wiley Periodicals). This article discloses an *in vitro* method for modifying the surface of endothelial cells of vascular tissue with PEG-biotin. In particular, the method comprises delivering NeutrAvidin-coated microspheres to PEG-biotin modified endothelial cells of bovine carotid arteries. The microspheres were found to adhere to the NHS-PEG-biotin treated bovine carotid arteries. Such specific targeting of a NeutrAvidin-coated entity demonstrates the ability of this method to find applications for drug delivery, gene therapy or cell therapy, where localization to specifically-modified vascular segments is desired, such as following catheter-based or vascular surgical procedures.

Based on the foregoing, Applicants respectfully request withdrawal of this rejection.

35 U.S.C. § 102 Rejection of Claims 1, 3, 5, 7, 10-12, 19, 23, 24, 26, 28 and 29

Claims 1, 3, 5, 7, 10-12, 19, 23, 24, 26, 28 and 29 are rejected under 35 U.S.C. § 102(b) as being anticipated by Pouletty et al. for the reasons set forth in the Office Action at page 11. Claim 28 has been canceled.

Applicants respectfully traverse this rejection and request that the rejection be reconsidered and withdrawn for the following reasons.

Contrary to the Examiner's assertion of anticipation of the claimed invention by Pouletty et al., Applicants point out that Pouletty et al. solely disclose introducing a reactive group into the blood stream to react with blood components circulating in the blood, such as red blood cells, immunoglobulins and proteins (column 2, lines 41-42), in order to provide functionalized blood components for the purpose of increasing the half-life of the functionalized blood components in the bloodstream (column 1, lines 60-62). Thus, an agent of interest can be maintained in the host bloodstream based on the lifetime of the blood component, i.e., the half-life of the blood component (column 1, line 65 and column 2, lines 8-10). Applicants submit that nowhere does Pouletty et al. disclose or suggest modifying the

surface of a segment of vascular tissue so as to allow for the targeted delivery of a chemical or biological entity to the surface of the vascular tissue segment. Indeed, the disclosure of Pouletty et al. is not directed to targeted delivery at all, but rather to introducing reactive groups into the bloodstream that generically link up with blood components in the bloodstream so as to functionalize the blood components and increase their concentration in the bloodstream. Applicants respectfully submit, therefore, that Pouletty et al. neither teaches nor suggests the claimed invention as claimed in claim 1 and new claim 30.

Dependent Claims 3, 5, 7, 10-12, 19, 23, 24, 26, 29, 31 and 32

Claim 3 recites the method of claim 1, wherein the covalent binding modifies the surface of the vascular tissue segment to provide a target for subsequent delivery of the chemical or biological entity. Applicants submit that nowhere does Pouletty et al. disclose or suggest that the covalent binding of the molecule recited in claim 1, from which claim 3 depends, modifies the surface of a segment of vascular tissue so as to allow for the targeted delivery of a chemical or biological entity to the surface of the vascular tissue segment.

Claim 11 recites the method of Claim 10, wherein the chemical entity is a pharmaceutical agent in a form selected from the group consisting of molecular, liposomal, micellar and particulate. Applicants submit that nowhere does Pouletty et al. disclose or suggest that their chemical is a pharmaceutical agent in a form selected from the group consisting of molecular, liposomal, micellar and particulate.

Claim 12 recites the method of Claim 11, wherein the pharmaceutical agent is an anti-thrombotic agent, an antimitotic agent, or a chemotherapeutic agent. Applicants submit that nowhere does Pouletty et al. disclose or suggest a pharmaceutical agent in a form selected from the group consisting of molecular, liposomal, micellar and particulate.

Claim 31 recites the method of claim 30, wherein the molecule further comprises polyethylene glycol and wherein the polyethylene glycol masks adhesive information inherent to the vascular tissue segment. Applicants submit that nowhere does Pouletty et al. disclose or suggest that the molecule recited in claim 30, from which claim 31 depends, comprises polyethylene glycol wherein the polyethylene glycol masks adhesive information inherent to the vascular tissue segment.

Claim 32 recites the method of claim 30, wherein the vascular tissue comprises endothelial cells. Applicants submit that nowhere does Pouletty et al. disclose or suggest vascular tissue comprising endothelial cells in the context of targeted delivery of drugs or cells to the surface of an endothelial vascular tissue segment for therapeutic purposes.

The features of dependent claims 5, 7, 10, 19, 23, 24, 26 and 29 are not asserted as independently establishing patentability apart from the claim or claims from which they depend. Applicants respectfully submit, therefore, that claim 1 as amended, and claims 3, 5, 7, 10-12, 19, 23, 24, 26 and 29, which depend either directly or indirectly from claim 1; and that new claim 30, and claims 31 and 32 which depend directly from claim 30, are not anticipated by Pouletty et al. Applicants respectfully request withdrawal of this rejection.

35 U.S.C. § 102 Rejection of Claims 1, 3, 5, 7, 10, 11, 13, 19, 23, 24, 28 and 29

Claims 1, 3, 5, 7, 10, 11, 13, 19, 23, 24, 28 and 29 are rejected under 35 U.S.C. § 102(e) as being anticipated by Bridon et al. for the reasons set forth at page 12 of the Office Action. Claim 28 has been canceled.

Contrary to the Examiner's assertion of anticipation of the claimed invention by Bridon et al., Applicants point out that Bridon et al. solely disclose methods and compositions for non-invasive imaging of mammals by using diagnostic agents modified to bond to proteins (page 1, paragraph [0006]). Specifically, Bridon et al. disclose diagnostic imaging agents, such as radioisotopes, which have been modified with reactive entities so that they will bond with reactive functionalities on blood components to provide increased half-lives for the diagnostic imaging agents (page 2, paragraph [0026]). Thus, in contrast to the claimed invention, which is directed to the targeted delivery of chemical or biological entities such as drugs or cells, to the surface of a vascular tissue segment for therapeutic purposes, the Bridon et al. disclosure solely is directed to targeting a diagnostic molecule, such as a radioisotope, to the vasculature of an organ for imaging purposes. Applicants respectfully submit, therefore, that Bridon et al. neither teaches nor suggests the claimed invention as claimed in claim 1 and new claim 30.

Dependent Claims 3, 5, 7, 10, 11, 13, 19, 23, 24, 29, 31 and 32

Claim 3 recites the method as described hereinabove. Applicants submit that nowhere does Bridon et al. disclose or suggest that the covalent binding of the molecule recited in claim 1, from which claim 3 depends, modifies the surface of a segment of vascular tissue so as to allow for the targeted delivery of a chemical or biological entity to the surface of the vascular tissue segment.

Claim 11 recites the method as described hereinabove. Applicants submit that nowhere does Bridon et al. disclose or suggest that their imaging agent is a pharmaceutical agent in a form selected from the group consisting of molecular, liposomal, micellar and particulate.

Claim 31 recites the method as described hereinabove. Applicants submit that nowhere does Bridon et al. disclose or suggest that the molecule recited in claim 30, from which claim 31 depends, comprises polyethylene glycol wherein the polyethylene glycol masks adhesive information inherent to the vascular tissue segment.

Claim 32 recites the method of claim 30, wherein the vascular tissue comprises endothelial cells. Applicants submit that nowhere does Bridon et al. disclose or suggest vascular tissue comprising endothelial cells in the context of targeted delivery of drugs or cells to the surface of an endothelial vascular tissue segment for therapeutic purposes.

The features of dependent claims 5, 7, 10, 13, 19, 23, 24 and 29 are not asserted as independently establishing patentability apart from the claim or claims from which they depend. Applicants respectfully submit, therefore, that claim 1 as amended, and claims 3, 5, 7, 10, 11, 13, 19, 23, 24 and 29, which depend either directly or indirectly from claim 1; and that new claim 30, and claims 31 and 32 which depend directly from claim 30, are not anticipated by Bridon et al. Applicants respectfully request withdrawal of this rejection.

35 U.S.C. § 103 Rejection of Claims 1-3, 5, 7-12, 19, 24 and 26-28

Claims 1-3, 5, 7-12, 19, 24 and 26-28 are rejected under 35 U.S.C. § 103(a) as being obvious over Pouletty et al. in view of Francis et al. and Kaiser et al. The Examiner asserts that Pouletty et al. are applied as described hereinbefore for claims 1, 3, 5, 7, 10-12, 19, 24, 26 and 28. Additionally, the Examiner asserts that Pouletty et al. do not teach a polymer, such as polyethylene glycol (PEG) that masks adhesive information inherent to the tissue or cellular surface (claims 2, 8 and 9), but that Francis et al. teach that PEG modification is a well-established technique that can solve or ameliorate many problems associated with protein pharmaceuticals and that PEGylation offers diverse advantages such as reducing toxicity and improving bioavailability. The Examiner also asserts that the motivation to PEGylate the molecules of the claimed invention is provided by Kaiser et al., who teach that PEG chains decrease nonspecific binding. In addition, the Examiner asserts that the limitation of local delivery of the chemical or biological entity is not innovative over the prior art.

As discussed hereinabove, Pouletty et al. solely disclose introducing a reactive group into the blood stream to react with blood components circulating in the blood, such as red blood cells, immunoglobulins and proteins, in order to provide functionalized blood components for the purpose of increasing the half-life of the functionalized blood components. Furthermore, nowhere does Pouletty et al. disclose or suggest modifying the surface of a segment of vascular tissue so as to allow for the targeted delivery of a chemical

or biological entity to the surface of the vascular tissue segment. Indeed, the disclosure of Pouletty et al. is not directed to targeted delivery at all, but rather to introducing reactive groups into the bloodstream that generically link up with blood components in the bloodstream so as to functionalize the blood components and increase their concentration in the bloodstream.

The disclosure of Francis et al., who teach that PEG modification is a well-established technique that can solve or ameliorate many problems associated with protein pharmaceuticals and that PEGylation offers diverse advantages such as reducing toxicity and improving bioavailability; and the disclosure of Kaiser et al., who teach that PEG chains decrease nonspecific binding, do not cure the deficiencies of Pouletty et al. Furthermore, there is no suggestion in Pouletty et al., Francis et al. or Kaiser et al. which would motivate one skilled in the art to combine these teachings, except with twenty-twenty hindsight, which is not a proper test for obviousness.

Applicants submit, therefore, that Pouletty et al., either alone or combination with Francis et al. and Kaiser et al., neither teaches nor suggests the claimed invention as claimed in claims 1-3, 5, 7-12, 19, 24 and 26-28. Applicants respectfully request withdrawal of this rejection.

35 U.S.C. § 103 Rejection of Claims 1, 3, 5, 7, 10, 11, 13, 19, 23-25, 28 and 29

Claims 1, 3, 5, 7, 10, 11, 13, 19, 23-25, 28 and 29 are rejected under 35 U.S.C. § 103(a) as being obvious over Bridon et al. in view of Palassis et al. The Examiner asserts that Bridon et al. are applied as described hereinbefore to claims 1, 3, 5, 7, 10, 11, 13, 19, 23, 24, 28 and 29. Additionally, the Examiner asserts that Bridon et al. do not teach a microbubble ultrasound contrasting agent (claim 25) but that Palassis et al. teach targeted delivery of ultrasound contrasting agents, in which the contrasting agent is a microbubble.

As discussed hereinabove, Bridon et al. solely disclose methods and compositions for non-invasive imaging of mammals by using diagnostic agents, such as radioisotopes, which have been modified with reactive entities so that they bond with reactive functionalities on blood components to provide increased half-lives for the diagnostic imaging agents. Thus, the Bridon et al. disclosure solely is directed to targeting a diagnostic molecule, such as a radioisotope, in which diagnostic molecules are targeted generally to the vasculature of an organ for imaging purposes, in contrast to the claimed invention, which is directed to the targeted delivery of chemical or biological entities, such as drugs or cells, to the surface of a segment of vascular tissue for therapeutic purposes. Furthermore, nowhere does Bridon et al.

disclose nor suggest the use of polyethylene glycol to block non-specific adhesion of their reactive entities.

The disclosure of Palassis et al., who teach targeted delivery of ultrasound contrasting agents, in which the contrasting agent is a microbubble, does not cure the deficiency of Bridon et al. Furthermore, there is no suggestion in either Bridon et al. or in Palassis et al. to combine the two teachings, and thus one skilled in the art would not be motivated to combine the two teachings other than with twenty-twenty hindsight, which, as described hereinabove, is not a proper test for obviousness.

Applicants submit, therefore, that Bridon et al., either alone or combination with Palassis et al., neither teaches nor suggests the claimed invention as claimed in claims 1, 3, 5, 7, 10, 11, 13, 19, 23-25 and 29. Applicants respectfully request withdrawal of this rejection.


In view of the foregoing amendments and remarks, it is respectfully submitted that claims 1-3, 5, 7-13, 19, 23-25-27 and 29-33 in the present application comply with the requirements of Section 112 and are patentable over the cited prior art. Accordingly, reconsideration and withdrawal of the rejections and an early Notice of Allowance are respectfully requested.

Respectfully submitted,



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Targeting microspheres and cells to polyethylene glycol-modified biological surfaces

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polyethylene glycol • targeted delivery • surface modification • biotin • avidin

ABSTRACT



It has previously been demonstrated that damaged arterial tissue can be acutely modified with protein-reactive polyethylene glycol (PEG) to block undesirable platelet deposition. This concept might be expanded by employing PEG-biotin and its strong interaction with avidin for site-specific targeted delivery. Toward this end, cultured endothelial cells (ECs) were surface modified with PEG-biotin and the available biotin was quantified with flow cytometry. NeutrAvidin-coated microspheres and PEG-biotin modified ECs with NeutrAvidin as a bridging molecule were delivered under arterial shear stress to PEG-biotin modified ECs on a coverslip as well as scrape-damaged bovine carotid arteries. After incubation with a 10 mM solution for 1 min, 8×10^7 PEG-biotin molecules/EC were found and persisted for up to 120 h.

Perfused microspheres adhered to NHS-PEG-biotin treated bovine carotid arteries with 60 ± 16 microspheres/mm² versus 11 ± 4 microspheres/mm² for control arteries ($p < 0.015$). Similarly, 22 ± 5 targeted ECs/mm² adhered to NHS-PEG-biotin treated bovine carotid arteries versus 6 ± 2 ECs/mm² for control arteries ($p < 0.01$). The targeting strategy demonstrated here might ultimately find application for drug delivery, gene therapy, or cell therapy where localization to specific labeled vascular regions is desired following catheter-based or surgical procedures. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 2006

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ARTICLE TEXT

INTRODUCTION



Polyethylene glycol (PEG) has been widely applied to modify surfaces or therapeutic proteins for biomedical applications. Attaching PEG molecules to peptides or proteins, commonly referred to as PEGylation, can provide a number of beneficial effects such as increased solubility, enhanced bioactivity, reduced immunogenicity, and improved pharmacokinetics.[1-3] Similarly, PEG modification of liposomes and microspheres inhibits nonspecific protein interactions and increases particle circulation time by slowing clearance from the blood.[4][5] PEG modification of synthetic surfaces is another common application where PEGylation can decrease protein adsorption and undesirable cell adhesion.[6-10] This approach has more recently been extended to tissue and cell surfaces to inhibit acute thrombosis and attenuate immune recognition.[11-15]

In the current study, *N*-hydroxysuccinimide-polyethylene glycol-biotin (NHS-PEG-biotin) was employed to covalently modify cellular and vascular surfaces. The active NHS ester of PEG reacts with primary amines, the most common being the epsilon amine of lysine, which can covalently link the PEG-biotin molecule to proteins found on cell membranes or extracellular matrix [Fig. 1(A)]. [17][18] This reaction can occur rapidly, within one minute, at physiologic temperature and pH. [19] As a result, labeling of tissue surfaces with protein-reactive PEG could prove invaluable in time-sensitive applications such as coronary procedures. Modification of vascular surfaces with protein-reactive PEG would be expected to provide a molecular barrier to prevent platelet and leukocyte adhesion [Fig. 1(B)] as demonstrated previously. [11][12] However, the addition of biotin to the terminus of the reactive PEG should provide a site for the targeted delivery of agents [Fig. 1(B)] using the high affinity between biotin and avidin ($K_a = 10^{15} M^{-1}$), which has been employed in a wide variety of detection, purification, and targeted delivery systems. [17][20-22] Specifically, the strong interaction between biotin and avidin has been employed for the targeted delivery of therapeutics in the treatment of malignant neoplasms. [20]

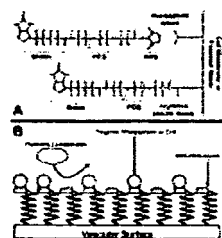


Figure 1. Illustration of the modification reaction for the protein-reactive polymer and schematic of the proposed targeted delivery system. A: Formation of the covalent bond between the protein-reactive polymer and the exposed amine on the vascular surface. B: The reactive polymer is covalently attached to the vascular surface forming a molecular barrier, inhibiting platelet and leukocyte adhesion. Targeted microspheres or cells will specifically adhere to vascular surfaces labeled with the polymer.
[Normal View 18K | Magnified View 40K]

In the system under investigation, we utilized NeutrAvidin instead of avidin. NeutrAvidin is a deglycosylated form of avidin with similar binding characteristics and lower nonspecific binding. NeutrAvidin-coated polystyrene microspheres were targeted to PEG-biotin modified vascular surfaces with the microspheres serving as a model for the delivery of particulate drugs, liposomes, or vesicles. Targeting cells was accomplished in a similar manner, but utilizing a two-step process where both cells and the vascular surface were modified with NHS-PEG-biotin. Bridging of the two biotinylated surfaces was accomplished by briefly incubating the biotinylated vascular surfaces with NeutrAvidin. Biotin and avidin mediated binding has previously been shown to enhance endothelial cell (EC) attachment, spreading, and a quiescent phenotype on synthetic surfaces. [23-26] These attributes may assist in targeting cells to vascular surfaces exploiting the strong interaction between biotin and avidin. Modification of vascular surfaces with protein-reactive PEG-biotin might thus provide a target for the site-specific delivery of microspheres and cells under physiologic flow conditions following intravascular procedures such as angioplasty or endarterectomy.

MATERIALS AND METHODS



Cell culture

Human coronary artery endothelial cells (HCAECs) (Cambrex, Walkersville, MD) were cultured in BD Falcon 75 cm² tissue culture flasks and 12 well tissue culture plates (Fisher Scientific, Pittsburgh, PA). In addition, HCAECs were cultured on 24 × 50 mm Gold Seal cover glasses in Fisherbrand 100 × 15 mm I-Plate compartmentalized petri dishes (Fisher Scientific, Pittsburgh, PA). ECs were grown in EGM-2 BulletKit medium composed of EBM-2 basal medium and SingleQuots with 5% FBS (Cambrex, Walkersville, MD).

Surface modification of ECs

Dry NHS-PEG-biotin (Nektar Therapeutics, San Carlos, CA) at -80°C was warmed to room temperature and phosphate buffered saline (PBS) was added to make 10 mM stock solutions. Aliquots of the stock solution were further diluted with PBS to make 5, 2.5, 1.25, and 0.625 mM solutions of the reactive polymer. Next, culture media on HCAECs in six wells of a 12 well plate were removed and replaced with 0.5 mL of 0, 0.625, 1.25, 2.5, 5, or 10 mM NHS-PEG-biotin solutions. The reactive polymer solutions were incubated on the cells for 1 or 5 min. After the desired incubation time, the reactive solutions were removed from the wells and the cells were washed three times with Dulbecco's Modified Eagle's Medium (DMEM) (Cambrex, Walkersville, MD). To measure PEG-biotin persistence on HCAECs, cells were incubated with 0.5 mL of the 10 mM NHS-PEG-biotin solution for 1 min. After this time, the solution was removed and the cells washed three times with DMEM. Cells were collected for flow cytometric analysis at time points of 0.5, 2, 4, 6, 9, 12, 24, 48, 72, 96, and 120 h with untreated HCAECs serving as a control.

Flow cytometric analysis of surface modification

Modified HCAECs were removed from the tissue culture plate with ReagentPack containing trypsin (Cambrex, Walkersville, MD) and individual samples collected in correspondingly labeled 16 × 75 mm round bottom polystyrene tubes (Fisher Scientific, Pittsburgh, PA). The tubes were centrifuged at 220g for 5 min and the supernatants removed. A 15 µL aliquot of a 5 mg/mL solution of fluorescein conjugated NeutrAvidin (Pierce Biotechnology, Rockford, IL) was diluted 1:40 with PBS and 100 µL of the solution added to each tube. Cell pellets were then resuspended in the tube by vortexing and allowed to incubate for 30 min at 4°C. After 30 min, the tubes were centrifuged at 220g for 5 min and the supernatants removed.

Cell pellets were resuspended and fixed with 500 µL of a 1% paraformaldehyde solution (Sigma, St. Louis, MO). Quantum FITC MESF High Level flow cytometry standard beads (Bangs Laboratories, Fishers, IN) were added to 500 µL of a 1% paraformaldehyde solution in another tube to allow for quantitative fluorescence determinations of the cell samples. [27] The intensity of the fluorescein conjugated NeutrAvidin signal for the cells modified with the varying PEG-biotin solution concentrations were gathered on a BD FACScan (San Jose, CA). The fluorescence intensity of the Quantum FITC MESF High Level beads was similarly collected to produce a calibration curve ($R^2 = 0.995-0.999$) and then determine the number of PEG-biotin molecules on the cell surface. [27]

Targeting microspheres and cells in a parallel plate chamber

HCAECs cultured on 24 × 50 mm Gold Seal cover glasses in Fisherbrand 100 × 15 mm I-Plate compartmentalized petri dishes were labeled with a 5 µM CellTracker Orange CMTMR (Molecular Probes, Eugene, OR) solution in EGM-2 media for 45 min in an incubator at 37°C. After

the incubation, the CellTracker Orange CMTMR solution was replaced with fresh EGM-2 media. Bovine serum albumin (BSA) (Sigma, St. Louis, MO) at 4.5 g/dL was added to DMEM and warmed to 37°C for the perfusion media.

The coverslip was loaded into a rectangular, parallel plate perfusion chamber to allow assessment of microsphere binding under physiologic flow conditions.[28] A silicon gasket and vacuum system secured the coverslip in the chamber creating an air-tight seal. The gap between the coverslip and top of the chamber was 200 μm and 2.65 cm^2 of the coverslip area was exposed to the perfusion media. Once the coverslip was loaded into the perfusion chamber, the system was first primed with media using a Harvard PHD 22/200 Infuse/Withdraw Syringe Pump (Harvard Apparatus, Holliston, MA) and then a 1.5 mL solution of 10 mM NHS-PEG-biotin or vehicle (PBS) was injected into the chamber through a stopcock and incubated for 1 min. After the incubation, the coverslip was perfused with media for 10 min.

A 4 μL aliquot of yellow-green fluorescent FluoSpheres NeutrAvidin labeled microspheres (Molecular Probes, Eugene, OR) was mixed with 200 μL of BlockAid Blocking Solution (Molecular Probes, Eugene, OR) and sonicated for 5 min to reduce nonspecific binding. The microsphere suspension was then further diluted to 10 mL with perfusate media to create a 7.28×10^6 microspheres/mL suspension that was drawn through the chamber at a controlled wall shear rate of 100 s^{-1} for 10 min followed immediately by another 10 min flush with the media alone. The number of adhered microspheres on the coverslip surface was counted following this perfusion using epi-fluorescence microscopy.

Similarly, HCAECs cultured on 24 \times 50 mm Gold Seal cover glasses in Fisherbrand 100 \times 15 mm I-Plate compartmentalized petri dishes were labeled with a 50 μM CellTracker Blue CMAC (Molecular Probes, Eugene, OR) solution in EGM-2 media for 45 min in the incubator. HCAECs grown in a BD Falcon 75 cm^2 tissue culture flask were also labeled with a 5 μM CellTracker Orange CMTMR solution in EGM-2 media for 45 min in the incubator. After the incubation, the CellTracker Blue CMAC and CellTracker Orange CMTMR solutions were replaced with fresh EGM-2 media.

Cells grown in the BD Falcon 75 cm^2 flask were removed from the flask using ReagentPack with trypsin and collected in a BD Falcon BlueMax Jr. 15 mL Graduated tube (Fisher Scientific, Pittsburgh, PA). The tube was centrifuged at 220g for 5 min and the supernatant removed. The cell pellet was dispersed and modified with 1 mL of a 10 mM NHS-PEG-biotin solution for 1 min. After modification, 9 mL of PBS was added to the cell suspension and the tube vortexed. Again, the cell suspension was centrifuged at 220g for 5 min and the supernatant removed. Next, the cells were resuspended in 10 mL of perfusate media and kept at 37°C in a Fisher Isotemp Economy water bath (Fisher Scientific, Pittsburgh, PA).

The same perfusate media, as was used for microsphere targeting, perfusion system, and wall shear rate was employed for the cell targeting experiments. Once the coverslip was loaded into the perfusion chamber, the system was primed with media using the syringe pump. Next, a 1.5 mL solution of 10 mM NHS-PEG-biotin or the vehicle (PBS) control was injected into the chamber through a stopcock and incubated for 1 min. After the incubation, the coverslip was perfused with media for 10 min and then 1.5 mL of a 2 mg/mL solution of NeutrAvidin Biotin-Binding Protein (Pierce Biotechnology, Rockford, IL) or vehicle (PBS) was injected into the chamber through a stopcock for another 1 min incubation. The coverslip was then perfused with media for 10 min followed by perfusion of the PEG-biotin modified cell suspension for 10 min and an immediate 10 min wash with the media alone. Finally, the number of adherent cells on the coverslip surface was counted using epi-fluorescence microscopy.

Targeting microspheres and cells to damaged arteries ex vivo

Fresh bovine carotid arteries were acquired and placed in a 90 mL specimen cup filled with PBS. Excess fat and tissue were removed from the outer surface of the artery and it was fileted along its length to expose the vessel lumen, which was damaged by scraping with a weighing spatula three times. Vessel segments measuring 2.5 cm in length were cut for use in a tubular perfusion chamber previously described for ex vivo perfusions.[29] The upper face of the tubular perfusion chamber had a cylindrical conduit with a window machined into the lower portion of the conduit to allow for introduction of test surfaces. The lower face of the tubular perfusion chamber possessed a raised surface, which was used to press and secure the sample into the window with clamps. Media was drawn through the chamber at a controlled wall shear rate of 100 s^{-1} .

For the microsphere targeting experiments, the scrape-damaged bovine carotid artery was loaded into the tubular perfusion chamber and the system was primed with media using the syringe pump. Next, a 1.5 mL solution of 10 mM NHS-PEG-biotin or vehicle (PBS) was injected into the chamber through a stopcock and incubated for 1 min. After the incubation, the artery was perfused with media for 10 min. A suspension of yellow-green FluoSpheres with NeutrAvidin labeling (7.28×10^6 microspheres/mL) was drawn through the chamber at the specified wall shear rate for 10 min followed immediately by another 10 min wash with the media alone. Finally, the number of bound microspheres on the damaged vessel surface was counted by inverting the vessel segment on a coverslip and utilizing epi-fluorescence microscopy.

For the cell targeting experiments, HCAECs grown in 75 cm^2 tissue culture flasks were labeled with CellTracker Orange CMTMR, harvested, and modified with NHS-PEG-biotin as described previously. The scrape-damaged bovine carotid artery was loaded into the tubular perfusion chamber and the system was primed with media using the syringe pump. Next, a 1.5 mL solution of 10 mM NHS-PEG-biotin or the vehicle (PBS) control was injected into the chamber through a stopcock and incubated for 1 min. After which, the artery was perfused with media for 10 min. Then, 1.5 mL of a 2 mg/mL NeutrAvidin solution or PBS vehicle was injected into the chamber through a stopcock for another 1 min incubation. After the incubation, the artery was perfused with media for 10 min. The PEG-biotin modified HCAEC suspension was then drawn through the chamber at a wall shear rate of 100 s^{-1} for 10 min followed immediately by another 10 min wash with the media alone. Finally, the number of adherent cells on the damaged vessel surface was determined using epi-fluorescence microscopy.

Statistical analysis

Data are presented as the mean \pm SEM. The number of PEG-biotin molecules per EC as a function of solution concentration and incubation time was compared using a Two-Factor ANOVA for equal sample sizes with $p < 0.05$ considered statistically significant. Control versus treated group comparisons for microsphere and cell targeting experiments were made using a Student's *t*-test with $p < 0.05$ being considered statistically significant.

RESULTS



Surface modification of ECs

The number of PEG-biotin molecules per EC determined from quantitative flow cytometry is shown as a function of reactive PEG solution concentration and incubation time in Figure 2. A maximum of 110 million PEG-biotin molecules per cell were observed with incubation in a 10 mM solution for 5 min. At an incubation time of 1 min, it was possible to bind ~80 million PEG-biotin molecules per EC. The number of PEG-biotin molecules per cell for each solution concentration tested was always greater after a 5 min incubation when compared to 1 min ($p < 0.001$). At the higher solution concentrations, with a 5 min incubation time, the number of PEG-biotin molecules per cell begins to plateau. Longer incubation periods were not evaluated due to their limited applicability, especially in coronary procedures. Also, demonstrated in Figure 2 is the covalent nature of the PEG attachment. If the *N*-hydroxysuccinimide active ester was removed by hydrolysis, no HCAEC modification was observed at 1 or 5 min. The duration of the PEG-biotin modification on the HCAEC surface at time points up to 120 h post-modification is shown in Figure 3. The number of PEG-biotin molecules decreases sharply over the first 24 h post-modification followed by a gradual decrease in the number of remaining molecules for the 120 h study duration. Over half (56%) of the initial number of PEG-biotin molecules per cell were lost in the first 12 h after modification with the reactive polymer. Moreover, just over 20% of the initial PEG-biotin modification persisted at 24 h post-modification. Finally, by the 120 h time point less than 1% of the initial number of molecules remained.

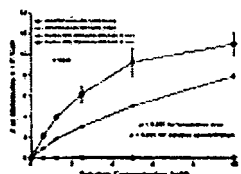


Figure 2. The number of PEG-biotin molecules reacted onto endothelial cell membranes as a function of solution concentration and incubation time. Cells were reacted with NHS-PEG-biotin solutions of 0, 0.625, 1.25, 2.5, 5, and 10 mM and incubation times of 1 or 5 min. The number of PEG-biotin molecules was determined using quantitative flow cytometry. The results are shown \pm SEM and are significant with $p < 0.001$ for the different solution concentrations and incubation times. [Normal View 9K | Magnified View 22K]

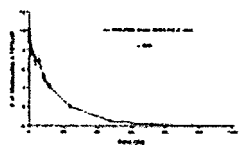


Figure 3. Persistence of PEG-biotin molecules on the cell surface over time. Cells were incubated with a 10 mM solution of NHS-PEG-biotin for 1 min. Samples were taken at 0.5, 2, 4, 6, 12, 24, 48, 72, 96, and 120 h. The number of PEG-biotin molecules was determined using quantitative flow cytometry and the results are shown \pm SEM. [Normal View 5K | Magnified View 10K]

Targeting microspheres and cells to cultured HCAECs

For microsphere targeting experiments, the cultured cells were labeled with CellTracker Orange CMTMR, which appear red, and targeted NeutrAvidin-coated microspheres appear green in Figure 4(A,B). The number of microspheres bound to PBS treated coverslips (10 ± 2 per mm^2) was significantly less ($p < 0.001$) than the number bound to NHS-PEG-biotin treated coverslips (115 ± 9 per mm^2) as demonstrated in Figure 4(C).

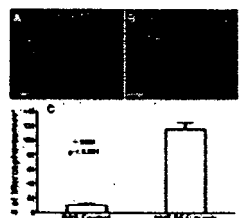


Figure 4. Targeted microspheres adhering to control and treated cells cultured on coverslips in a parallel plate chamber. A: Cultured cells (red) on a coverslip treated with the vehicle (PBS) and then perfused with targeted microspheres (green). B: Cells (red) cultured on a coverslip treated with 10 mM NHS-PEG-biotin and then perfused with targeted microspheres (green). Scale bar is 100 μm . C: Number of adherent microspheres per mm^2 on control and PEG-biotin treated coverslips. Data is shown \pm SEM and the results are significant with $p < 0.001$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.] [Normal View 28K | Magnified View 78K]

In the targeted cell delivery experiments, the cultured HCAECs on the coverslip were labeled with CellTracker Blue CMAC and appear this color in Figure 5(A,B); whereas, the targeted HCAECs that deposited from flow were labeled with CellTracker Orange CMTMR and appear red. The number of targeted cells bound to NHS-PEG-biotin treated coverslips (20 ± 5 cells per mm^2) was significantly greater ($p < 0.005$) than the number bound to PBS treated coverslips (1 ± 0.3 cells per mm^2) by ~20-fold [Fig. 5(C)].

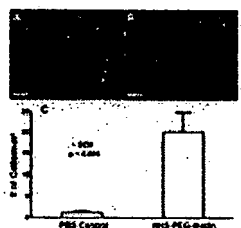


Figure 5. Targeted cells adhering to control and treated cells cultured on coverslips in a parallel plate chamber. A: Cultured cells (blue) on a coverslip treated with the vehicle (PBS) and then perfused with targeted cells (red). B: Cells (blue) cultured on a coverslip treated with 10 mM NHS-PEG-biotin, 2 mg/mL NeutrAvidin, and then perfused with targeted cells (red). Scale bar is 100 μm . C: Number of adherent cells per mm^2 on control and PEG-biotin treated coverslips. Data is shown \pm SEM and the results are significant with $p < 0.005$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.] [Normal View 27K | Magnified View 71K]

Targeting of microspheres and cells to damaged arteries ex vivo

In the images of microsphere adherence to scrape-damaged bovine carotid artery segments, the arteries exhibited background auto-fluorescence in green [Fig. 6(A,B)]. Similarly, targeted NeutrAvidin-coated microspheres were yellow-green fluorescent; however, the fluorescence intensity of the microspheres was identifiable over the background auto-fluorescence of the vessel. For clarity, the fluorescent microspheres are presented in purple pseudo-color [Fig. 6(A,B)]. The number of microspheres bound to untreated damaged bovine carotid arteries was significantly less ($p < 0.015$) than the number bound to NHS-PEG-biotin treated arteries, 11 ± 4 per mm^2 versus 60 ± 16 per

mm² [Fig. 6(C)].

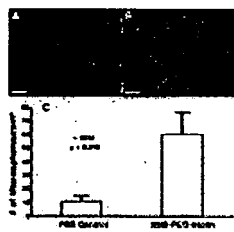


Figure 6. Targeted microspheres adhering to control and treated scrape-damaged bovine carotid arteries in a tubular flow chamber. **A:** Scrape-damaged bovine carotid artery (green) treated with the vehicle (PBS) and then perfused with targeted microspheres (purple). **B:** Injured bovine carotid artery (green) treated with 10 mM NHS-PEG-biotin and then perfused with targeted microspheres (purple). Scale bar is 100 μ m. **C:** Number of adherent microspheres per mm² on control and PEG-biotin treated scrape-damaged bovine carotid arteries. Data is shown + SEM and the results are significant with $p < 0.015$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.] [Normal View 22K | Magnified View 60K]

In examining HCAEC adhesion from shear onto PEG-biotin treated scrape-damaged arteries, targeted cells appear red on the auto-fluorescent vessel background [Fig. 7(A,B)]. The number of targeted cells bound to NHS-PEG-biotin treated bovine carotid arteries (22 ± 5 per mm²) was significantly greater ($p < 0.01$) than the number bound to PBS treated arteries (6 ± 2 per mm²) as shown in Figure 7(C).

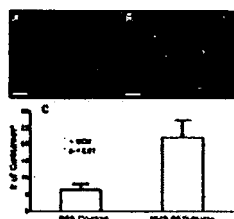


Figure 7. Targeted cells adhering to control and treated scrape-damaged bovine carotid arteries in a tubular flow chamber. **A:** Scrape-damaged bovine carotid artery (green) treated with the vehicle (PBS) and then perfused with targeted cells (red). **B:** Injured bovine carotid artery (green) treated with 10 mM NHS-PEG-biotin, 2 mg/mL NeutrAvidin, and then perfused with targeted cells (red). Scale bar is 100 μ m. **C:** Number of adherent cells per mm² on control and PEG-biotin treated scrape-damaged bovine carotid arteries. Data is shown + SEM and the results are significant with $p < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.] [Normal View 17K | Magnified View 46K]

DISCUSSION



The experimental data demonstrate the feasibility of rapidly modifying vascular or endothelial surfaces with protein-reactive PEG-biotin under physiologic conditions for subsequent targeting of microspheres or modified ECs. Even without the targeting biotin molecule, modification of injured vascular surfaces with PEG might provide benefit in preventing platelet deposition following coronary interventions. It has previously been shown that PEG molecular barriers (without biotin) on damaged vascular surfaces can inhibit acute thrombotic deposition.[11][12] Several other studies have also employed surface modification of vascular tissue to reduce thrombosis at the sites of vascular injury.[30][31] The effectiveness of the polymer coating to serve as a molecular barrier to deposition or anchor for targeting to biotin on biological tissues would depend upon the density and structure of the PEG molecules on the modified surface. PEG molecules might assume pancake, mushroom, or brush conformations depending upon the distance between the covalently linked molecules and the properties of the surface.[32][33] Based upon calculations for the expected structure, the number of PEG-biotin molecules per EC observed in this study would occupy 87% or 6% of the cell surface if the PEG molecules were in the mushroom or brush conformation, respectively.[32][33]

A potential benefit to using the approach of this study would be the ability to target pharmaceuticals or vectors to specific sites within the vasculature labeled with PEG-biotin for therapeutic benefit. Delivery of pharmaceutical agents to sites of vascular injury is another strategy to reduce acute platelet deposition and possibly inhibit intimal hyperplasia. For example, in one recent report anti-restenotic agents, heparin and low molecular weight heparin, were conjugated to antibodies against cross-linked fibrin.[34] These modified antibodies were then systemically injected where they putatively targeted to the site of arterial injury.[34] The targeting of heparin in this manner was shown to reduce neointimal formation in balloon damaged rabbit carotid arteries. Pharmaceutical targeting to the PEG-biotin labeled site might also allow the localization of anti-proliferative agents for an effect similar to that reported by Thomas and Campbell.[34] The protein-reactive PEG targeting approach is also advantageous because of its applicability to healthy endothelium or surfaces where fibrin might not be present, for delivery to locations selected by the clinician at the time of the intervention.

Another approach investigated as a means of inhibiting restenosis following vascular injury seeks to facilitate re-endothelialization of the damaged vessel luminal surface.[35-37] Disruption of the endothelial layer following various intravascular procedures is thought to be a major factor in thrombotic occlusions and neointimal formation.[38][39] Accelerated replacement of the endothelial layer might abrogate thrombus formation and platelet deposition, as well as act to down-regulate smooth muscle cell proliferation, thereby reducing restenosis. In one study, previously harvested ECs with fibroblast growth factor type 1 (FGF-1) and heparin were delivered to lumens of balloon injured arteries using fibrin glue.[37] Administration of the ECs, FGF-1, and heparin in the fibrin glue was associated with an inhibition of neointimal thickening, but required a 8 min incubation for polymerization and an additional step to remove the excess glue.[37] Other studies have focused on harvesting endothelial progenitors and incubating them inside the vessel following injury.[35][36] The attachment times investigated with the endothelial progenitor incubation technique were 20 min or greater, which would not be well-suited to time sensitive procedures, such as those involving coronary arteries.

The approach with the protein reactive PEG-biotin, where PEG is attached to the vessel lumen for a 1-min period followed by a similar NeutrAvidin incubation period, could be brief enough for consideration of this technique *in vivo* as a method for directing cells from the circulation to deposit at sites of modification where extended access is not feasible. A constraint inherent in this technique is the requirement that the targeting cells must first be isolated and surface modified with PEG-biotin. A theoretical benefit would be the potential to direct cells to sites selected by the localized application of the protein-reactive PEG-biotin by the clinician. This could be readily accomplished through the use of specialized local delivery balloon catheters, which are designed to allow fluid delivery to a specific region of the blood vessel wall.[40][41] Further work would be necessary to characterize the phenotypic behavior and proliferation potential of the ECs following deposition

in vivo. We have previously demonstrated that treatment of ECs with a reactive PEG solution resulted in no change in cell viability or proliferation potential when compared to untreated controls at 2, 6, and 9 days post-exposure.[12] Furthermore, microscopic visualization of PEG-biotin modified cells displayed no evidence of membrane fusion or a decrease in viability or proliferation potential in the current study.

Several limitations associated with the current report should be noted. Perhaps of greatest significance are the limits associated with the use of *in vitro* or *ex vivo* models that employed relatively brief perfusions of modified cell culture medium containing the targeted species. Blood components were not present in the perfusate, and although albumin was added, the numerous plasma proteins and cells that would be encountered *in vivo* were absent. The perfusion periods were single pass and relatively short (10 min). *In vivo* studies, or employment of a recirculation loop, would have allowed longer contact times. An increased perfusion time might have led to higher deposition levels for the microspheres and cells, which on an absolute scale were somewhat sparse in the reported experiments. Deposition might also have been increased by not relying entirely upon adhesion from flow, but by employing some period of stagnant incubation. The deposition from flow model was favored, however, since it was believed to have the broadest clinical relevance and would be the more stringent test of the concept.

Another potential limitation of the targeted delivery system could be the loss of the PEG-biotin from the modified surfaces over time. This loss is postulated to be a result of natural cellular processes and not due to cytotoxicity, since the latter was not observed in previous studies PEGylating ECs directly, as mentioned previously.[12] The reduction in attached surface molecules would also be expected to occur *in vivo* for damaged vascular surfaces as healing ensues. The time frame of PEG-biotin loss observed in this report suggests that substantial numbers of PEG-biotin may remain on cellular surfaces for periods of one day, or possibly longer, depending on the surface concentration needed to facilitate targeting. The concentration effect on targeting efficiency remains to be investigated.

In conclusion, we have demonstrated that a protein-reactive PEG-biotin could be used to modify damaged vascular tissue or cultured ECs *in vitro* such that it was possible to target microspheres and cells to the modified surfaces under flow conditions. This approach might ultimately find application in the targeting of pharmaceutical agents or cells to vascular sites selected by the controlled application of the protein-reactive PEG-biotin. Such targeted delivery could be of value in vascular interventions where thrombosis and restenosis are of concern, as well as to conditions where it is desirable to target an agent or cell to a user-specified location, such as tumor vasculature or regions where stem cell localization is desirable.

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